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ASSOCIATION OF CENTRIOLES WITH THE MARGINAL BAND IN SKATE ERYTHROCYTES

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Abstract

Centrioles are associated with the marginal bands (MBs) in certain invertebrate erythrocytes, functioning as organizing centers for MB reassembly (Nemhauser *et al.*, 1983). However, a similar association has not been observed previously in erythrocytes of vertebrates. Detergent-lysed erythrocytes ("cytoskeletons") of the skate *Raja erinacea* contain centrioles visible as paired dense dots in phase contrast. In uranyl acetate-stained whole mounts and in thin sections (TEM) they exhibit typical right-angle orientation and 9-triplet ultrastructure. Although the centriole pairs in some cytoskeletons are distant from the MB, surveys of their distribution in preparations from different animals indicates that it is non-random, with the majority adjacent to the MB or less than 1 μm from it. Many of the centriole pairs appear to be attached to MB microtubules, or have microtubules extending from them toward the MB. In rare instances, pointed cytoskeletons are observed with the centrioles at the apex from which fibers radiate, suggesting a morphogenetic function. The observations support the possibility that centrioles function during MB biogenesis in differentiating vertebrate erythrocytes, with loss of functional location as the cells mature.

Introduction

Comparative studies of cytoskeletal structure in blood cells of vertebrates and invertebrates have shown that marginal bands (MBs) of microtubules are prominent components of erythrocytes and clotting cells throughout the animal kingdom (Meves, 1911; Fawcett and Witebsky, 1964; Behnke, 1970; Goniakowska-Witalinska and Witalinksi, 1976; Cohen and Nemhauser, 1985). In erythrocytes, MBs first appear during cellular morphogenesis and are believed to function in bringing about the transformation from spherical to flattened discoid or elliptical cell shape (Barrett and Scheinberg, 1972; Small and Davies, 1972; Barrett and Dawson, 1974; Yamamoto and Iuchi, 1975). Recent experiments on vertebrate and invertebrate erythrocytes indicate that MBs continue to function in mature cells, resisting shape changes and/or restoring cell shape after deformation by external forces (Joseph-Silverstein and Cohen, 1984, 1985).

Although the circumferential location of MB microtubules in the plane of cell flattening is of considerable interest with respect to spatial control of microtubule arrays, relatively little is known of the mechanisms involved in MB formation. In mature chicken erythrocytes there may be structural or molecular "tracks" along the inner cell surface which guide the growth of MB microtubules during experimentally

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Abbreviations: PIPES = piperazine-n-n'-bis (2-ethane sulfonic acid), EGTA = ethyleneglycol-bis-(b-aminoethyl ether) n n'-tetraacetic acid, TAME = p-tosyl arginine methyl ester HCl, MB = marginal band, SAC = cell surface-associated cytoskeleton, PMSF = phenylmethylsulfonyl fluoride.

induced MB reassembly (Granger and Lazarides, 1982; Miller and Solomon, 1984), and in chick bone marrow erythroblasts centrosomes may play a role at an early stage of MB formation (Murphy *et al.*, 1986). In certain invertebrate ("blood clam") erythrocytes, MB-associated centrioles serve as organizing centers during temperature or taxol-induced MB reassembly (Cohen and Nemhauser, 1980; Nemhauser *et al.*, 1983; Joseph-Silverstein and Cohen, 1985), and MB-associated centrioles have also been observed in sea cucumber erythrocytes (Fontaine and Lambert, 1973). However, a similar structural relationship has not been reported previously for mature (circulating) erythrocytes of any vertebrate.

The purpose of this paper is to show that, for one vertebrate at least, such an association does exist. In the skate, *Raja erinacea,* most of the erythrocytes contain readily identifiable centriole pairs, a majority of which are adjacent to the MB.

Materials and Methods

Skates (*Raja erinacea*) were provided by the Department of Marine Resources of the Marine Biological Laboratory, Woods Hole, Massachusetts. The animals were maintained in running seawater at MBL or in cooled (10°C), aerated tanks of "Instant Ocean" artificial seawater (Aquarium Systems, Inc., Eastlake, Ohio) at Hunter College. In the latter case "trace elements" (Hawaiian Marine Imports, Inc., Houston, Texas) were added to the water periodically.

Small samples of blood were obtained by snipping the tail tip, and larger ones by heart puncture after anesthesthetizing the animals in 0.04% tricaine in seawater. "Cytoskeletons" for routine examination and scoring of centriole location under phase contrast, were prepared by dilution of blood or cell suspensions approximately 1:10 into Triton lysis medium consisting of 100 m*M* PIPES, 5 m*M* EGTA, 1 m*M* $MgCl_2$, pH 6.8 (=PEM) containing 10 m*M* TAME and 0.4% Triton X-100. This medium had been used previously for studies of cytoskeletal structure in blood cells of diverse species (Cohen, 1978; Cohen *et al.*, 1982; Cohen and Nemhauser, 1985). In one experiment, 0.1% glutaraldehyde was included in the lysis medium to achieve simultaneous lysis and fixation (Cohen and Nemhauser, 1980). Cell morphology was observed in phase contrast in both living cells and cells fixed in Elasmobranch Ringer's (Cavanaugh, 1975) containing 0.1% glutaraldehyde so as to avoid artifacts due to contact with glass slides and coverslips.

Cytoskeleton whole mounts for transmission electron microscopy were prepared on Formvar-coated grids. The Formvar surface was pre-treated with polylysine (1% solution of MW > 400,000, followed by water washes and air drying) to enhance retention of material (Mazia *et al.*, 1975). Cytoskeleton suspensions in Triton lysis medium were placed on grids for 5 min, followed by a wash in PEM and 10 min fixation in PEM containing 2% glutaraldehyde. Subsequently, grids were washed in PEM and in water, stained with 1% aqueous uranyl acetate, and air-dried.

Material was prepared for thin sectioning as follows: 0.3 ml packed washed cells were suspended in 6 ml Brij lysis medium, consisting of PEM containing 0.6% Brij 58, 10 m*M* TAME, and 0.1 m*M* PMSF (freshly added). Brij was used because it produced less twisting of cytoskeletons than Triton, and also permitted easy resuspension of cytoskeletons. The material was centrifuged for 1 minute at top speed in the International Clinical centrifuge (2250 × *g*), resuspended as before and centrifuged again. The cytoskeletons were resuspended and fixed 1 h at room temperature in 6 ml PEM containing 2.5% glutaraldehyde. They were sedimented, washed once in 6 ml PEM by resuspension and centrifugation, postfixed for 1 h in 1% OsO_4 in PEM, washed three times in PEM, dehydrated in ethanol, and embedded in Epon.

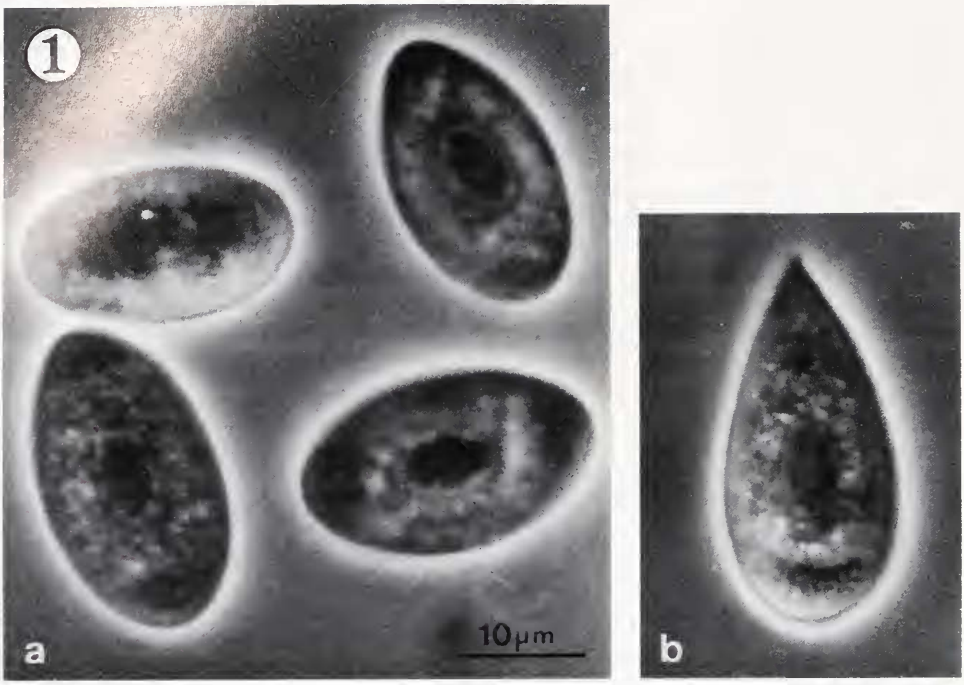

FIGURE 1. Erythrocytes of the skate, *Raja erinacea,* exhibiting the nucleated, flattened, elliptical morphology typical of all non-mammalian vertebrates (a). On rare occasion, cells with single or double-pointed shape are observed (b). Phase contrast.

Thin sections were cut with diamond knives on the Sorvall MT-2 ultramicrotome (DuPont Instruments, Newtown, Connecticut), and stained with saturated uranyl acetate in 50% ethanol followed by Reynold's lead citrate. Whole mounts and thin sections were examined in the Hitachi HS-8 (50 kV) or Zeiss EM10C (80 kV) transmission electron microscopes.

RESULTS

In both fresh blood samples and samples of washed, fixed cells, the erythrocytes of the skate, *Raja erinacea,* are found to be morphologically similar to those of most non-mammalian vertebrates (Fig. 1a). They are nucleated, flattened, and, with rare exception, elliptical, with the long axis in the 20–25 μm range. This is a relatively large size for fish in general, but typical of elasmobranchs as compared with teleosts (Andrew, 1966; Nemhauser *et al.,* 1979). Cells with single or double-pointed shape are rarely observed morphological variants (Fig. 1b).

Skate erythrocyte cytoskeletons, prepared by lysis of the cells with Triton X-100 under conditions previously observed to stabilize MBs, are shown in Figure 2. In phase contrast the centrioles, verified as such by electron microscopy (see below), appear as paired dense "dots," and usually they are readily visible in flat (untwisted) cytoskeletons because there is little competing cytoplasmic structure. In many cases the centrioles are very close to and possibly in contact with the MB. At higher magnifications, with flattening of cytoskeletons under the coverslip to enhance viewing,

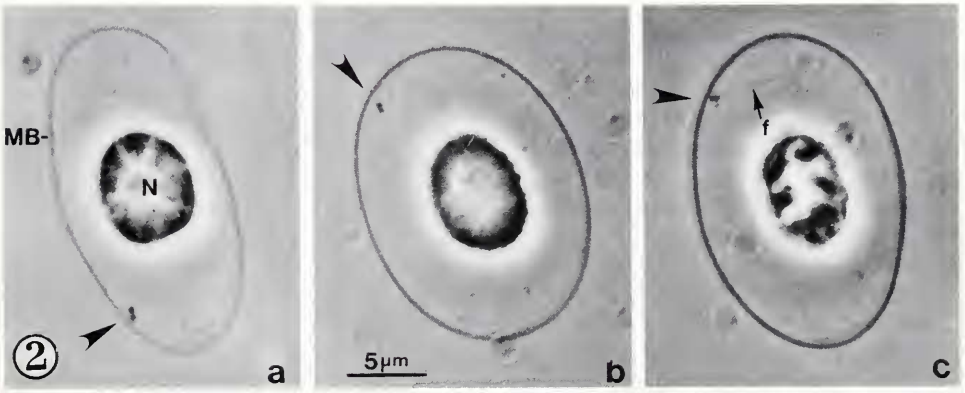

FIGURE 2. Skate erythrocyte cytoskeletons (Triton X-100 lysis), as observed in phase contrast under oil immersion. Centrioles appear as paired, phase-dense "dots" (arrowheads). (a) One of the centrioles very close to or in contact with MB; N = nucleus. (b) Centriole pair adjacent to, but not in direct contact with major part of MB; (c) centriole pair appearing to touch MB, with a "fiber" (f) extending away from it.

some of the centriole pairs are found to be attached to fibers (microtubule bundles) which are part of the MB, or which extend from the centrioles toward a distant point on the MB (Fig. 3).

Confirmation of the attachment of centrioles to the MB, including some which appeared separated from the main body of the MB, was obtained by examination of cytoskeleton whole mounts in TEM. In Figure 4 the centrioles are observed to be attached to only one or possibly a few microtubules of the spread MB. Figure 5 shows centrioles which, in phase contrast, would appear to be close to, but not touching the MB, but which are actually connected to it by radiating microtubules. Examination of the centrioles in underexposed prints revealed their cylindrical shape, microtubular substructure, and sometimes (as in Fig. 5) their approximately orthogonal orienta-

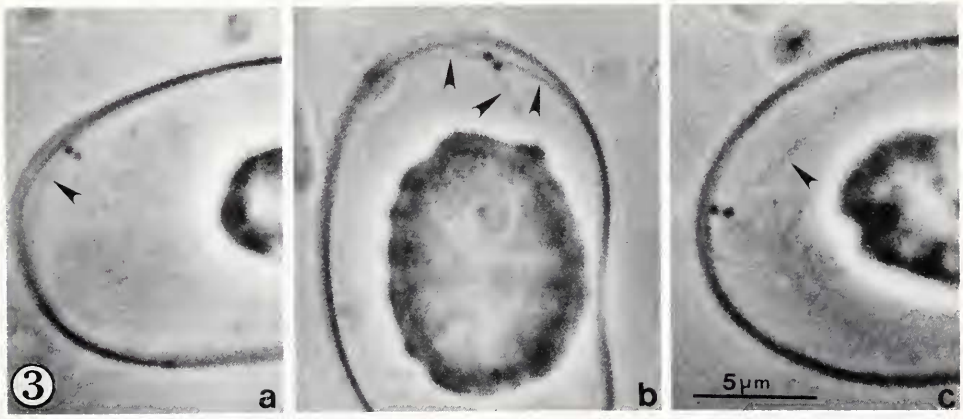

FIGURE 3. Cytoskeletons flattened under the coverslip to provide improved higher magnification views of centriole-associated fibers in phase contrast. Such fibers appear to be part of the MB (a, b; arrowheads), or to extend from at least one of the centrioles toward a distant point on the MB (c; arrowhead). Flattening also generally produced an artifactual increase in area of nucleus, as in b.

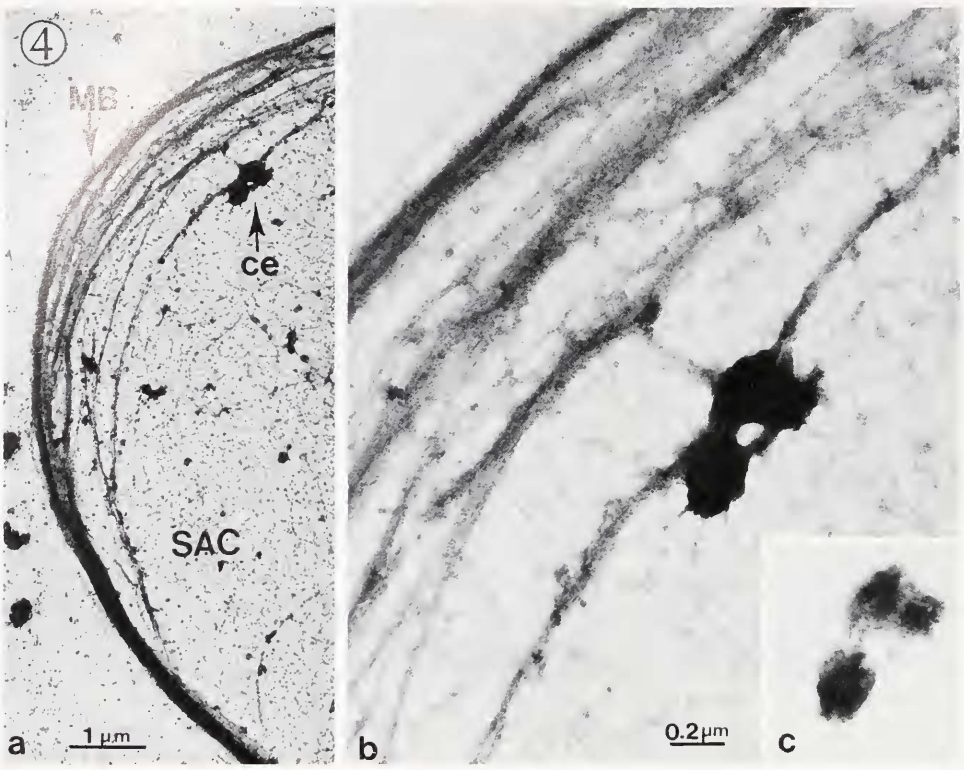

FIGURE 4. Skate erythrocyte cytoskeleton whole mount, uranyl acetate staining, TEM. (a) Survey view; the MB in this region has spread against the substratum; centrioles = ce. The "membrane skeleton," or "cell surface-associated cytoskeleton" (SAC), has collapsed onto the substratum and is visible as a background network. (b) Higher magnification view of centrioles in (a), revealing that the centriole pair is attached to one, or at most a few, MB microtubules. (c) Underexposed print of centrioles in b, in which centriolar substructure can be detected.

tion. Thin sections confirmed that they were typical centrioles, about 0.2×0.35 μm, with "9 + 0" substructure (Fig. 6).

While most cytoskeletons contained centrioles adjacent to or near the MB, in some the centrioles were located between MB and nucleus, while in others they were adjacent to the nucleus. Figure 7 illustrates a case in which the centriole pair was closer to nucleus than to MB. In such cases, radiating microtubules usually were not evident. In all of the whole mounts examined by TEM, the cell "membrane skeleton" or "surface-associated cytoskeleton" (SAC) was visible as a background network throughout the region between nucleus and MB (Figs. 4, 5, 7).

Two cytoskeletons out of several thousand examined in phase contrast were pointed and incomplete at one end, with a curved MB at the other end. In these, the centrioles were located within the pointed tip, in a region from which fibers radiated toward the closed end of the MB (Fig. 8). Though rare, these cytoskeletons gave the impression that the centrioles were active at one "pole" as organizing centers for a forming MB.

Upon casual inspection, the spatial distribution of centrioles appeared to be nonrandom, with most centriole pairs close to or touching the MB. This was confirmed

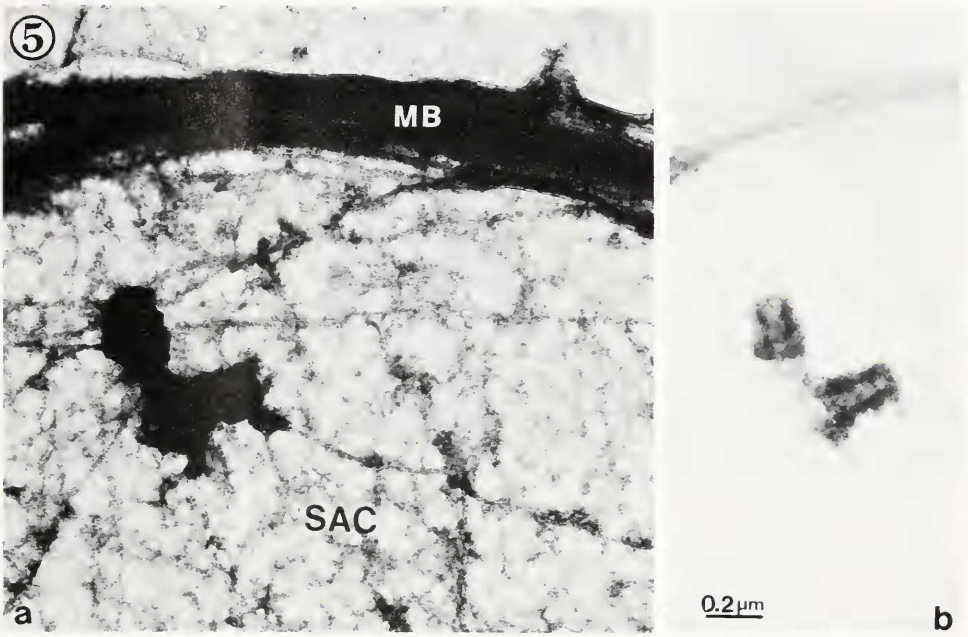

FIGURE 5. Skate erythrocyte cytoskeleton whole mount, uranyl acetate staining, TEM. (a) Survey view; the MB in this region is compact (not spread as in Fig. 4), with radiating microtubules connecting the centriole pair to the MB. The cell surface-associated cytoskeletal network (SAC) also is visible. (b) Underexposed print of the centrioles, showing cylindrical structure and nearly right-angle orientation.

in a semi-quantitative way by careful oil immersion observation of large numbers of cytoskeletons from six animals (Table I). A tabulation procedure was devised to count MB-associated *versus* cytoplasmic centriole pairs, so as to provide a general picture of their distribution (Fig. 9). For each of the six skates, 50% or more of the centriole

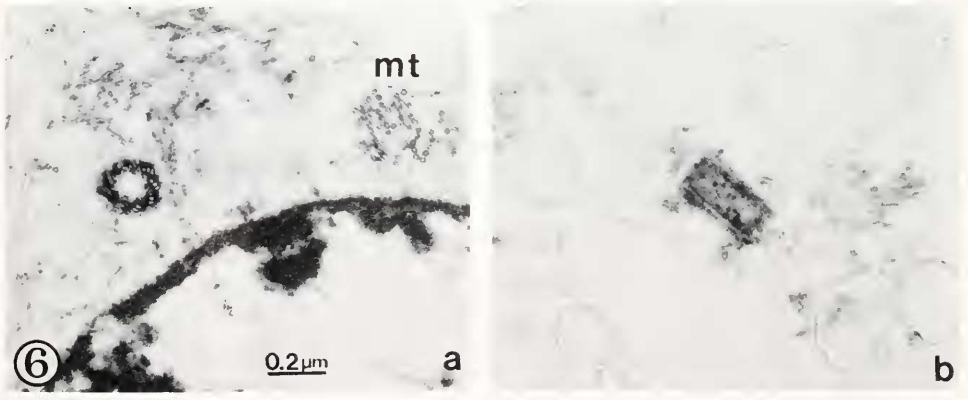

FIGURE 6. Skate erythrocyte centrioles as seen in thin sections of cytoskeletons, TEM. Typical "9 + 0" cross-sectional pattern (a) and cylindrical longitudinal structure (b) is observed. MB microtubules are also evident (mt).

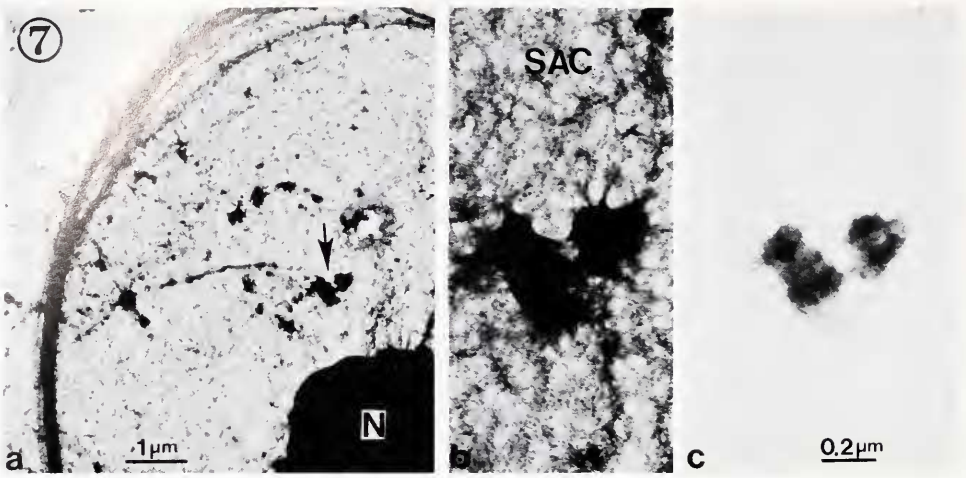

FIGURE 7. Skate erythrocyte cytoskeleton whole mount, uranyl acetate staining, TEM. Example in which the centriole pair is located closer to nucleus than to MB. (a) Survey view; centrioles at arrow; N = nucleus. (b) Higher magnification view of centrioles; few, if any, radiating microtubules are present. The centrioles are enmeshed between the two surface-associated cytoskeleton layers, which form a surrounding network (SAC). (c) Underexposed print of the centriole pair.

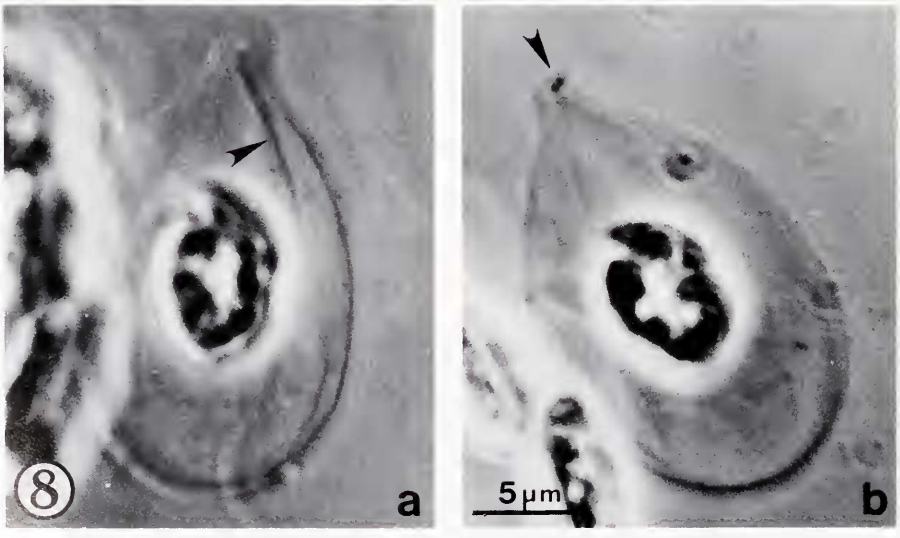

FIGURE 8. A rarely observed pointed skate erythrocyte cytoskeleton, as viewed in phase contrast under oil immersion. (a) One of several fibers (arrowhead) emanating from pointed (upper) region of cytoskeleton. These fibers radiate toward the distant closed end of the MB. (b) Different optical section of same cytoskeleton, showing the pair of centrioles present at the apex of this pointed region (arrowhead). (Note: material at left in photos is part of adjacent clump of cytoskeletons, and different orientation of cytoskeleton in b is due to its movement under coverslip between photographs.)

TABLE I

Distribution of centrioles in erythrocyte cytoskeletons prepared from different animals (phase contrast observations, oil immersion)

Skate no.[a]	No. cytoskeletons counted[b]	No. with centrioles adjacent to MB[c]	% Adjacent
1	104	70	67
2	107	65	60
3	100	80	80
4	100	52	52
5	103	52	50
6	100	55	55

[a] Blood samples were taken from skates during summer months at the MBL, except for #5, which was maintained at Hunter College at 10°C without feeding for more than two months before use.

[b] Triton lysis medium was used to prepare cytoskeletons for skates #1–5. For skate #6, Triton lysis medium containing glutaraldehyde was employed to achieve simultaneous lysis and fixation (see Materials and Methods).

[c] Criterion: at least one centriole of the pair within or touching outer zone of cytoskeleton (see Fig. 9).

pairs were within or touching an "outer zone" adjacent to the MB, representing less than 25% of the open area (excluding nucleus) of a cytoskeleton. Since the surface of these cells (as viewed on edge) tends to taper as it approaches the narrow perimeter containing the MB, the *volume* represented by the "outer zone" (Fig. 9) is actually less than 25% of that available. It was notable that in one of the skates, 80% of the centriole pairs were partially or wholly within this area (#3, Table I).

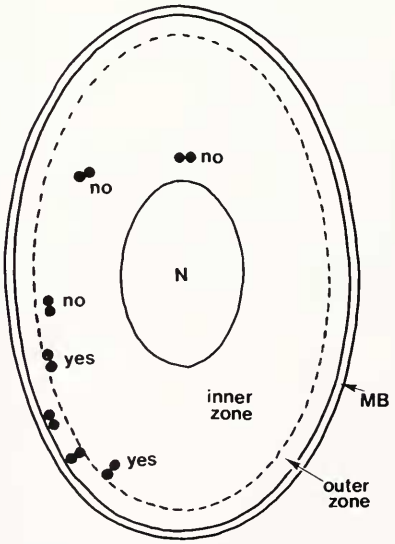

FIGURE 9. Diagram illustrating the counting procedure used to determine centriole distribution as reported in Table I. In addition to centrioles in contact with the MB, those within or touching the "outer zone" of a cytoskeleton were scored as adjacent to the MB (examples labelled "yes"); those in the interior ("inner zone") were scored as not MB-associated (examples labelled "no"). The "outer zone" was approximately one centriole pair (0.75 μm) in width, representing <25% of open area within MB (excluding nuclear area).

Discussion

Centrioles have been observed previously in the mature erythrocytes of several non-mammalian vertebrates, including amphibia (*Xenopus laevis, Rana catesbiana, Necturus maculosus*) and a reptile (*Stenotherus odoratus*) (Gambino *et al.,* 1982; Euteneuer *et al.,* 1985), and data on microtubule polarity in MBs of several of these species are consistent with a model involving initiation of microtubule growth at an organizing center (Euteneuer *et al.,* 1985). However, the centrioles are generally adjacent to the nucleus rather than associated with MBs in these vertebrates, consistent with the idea that MB biogenesis in vertebrate erythrocytes may involve other kinds of organizing centers and/or spatial determinants, such as peripheral morphological and/or molecular "tracks" (Granger and Lazarides, 1982; Miller and Solomon, 1984; Swan and Solomon, 1984).

The present observations on skate erythrocytes suggest a relationship between centrioles and MBs, raising the issue as to why significant numbers of MB-associated centrioles have not been observed in other vertebrates. One possibility is that the skate, as a relatively primitive, cartilagenous vertebrate, retains some primitive traits at the cellular level which have been lost in higher vertebrates. This is unlikely, because in the dogfish (their close elasmobranch relative) we have observed MB-associated centrioles relatively infrequently (Nemhauser *et al.,* 1979). Another possibility is that such an association is seasonal, disappearing except during periods of maximal erythropoiesis for a given species. The current study does not explore this question. Blood samples from skates #1–4 were obtained during the summer at a time when heightened growth and erythropoiesis might be expected; skate #5 (Table I) was maintained for >2 months at 10°C without feeding, yet its erythrocytes retained roughly 50% of centrioles near the MB. However, the significance of this observation cannot be determined at present, since it is unlikely that the treatment of skate #5 was physiologically equivalent to normal seasonal changes experienced in nature.

In considering the data on centriole distribution (Table I), it should be noted that centrioles cannot be studied readily by light microscopy in intact living or fixed erythrocytes, principally because they are obscured by hemoglobin. In addition, immunofluorescence techniques are not useful in fixed intact erythrocytes because the fixed hemoglobin forms a gelled matrix through which both antibody penetration and viewing are poor. Thus, examination of detergent-lysed cells (cytoskeletons) is the only convenient approach to studying large numbers of these cells. Such work requires the assumption that the distribution of centrioles in erythrocyte cytoskeletons reflects their location in living cells. The arguments that this is the case are as follows: (a) many of the skate erythrocyte centriole pairs are immediately adjacent to the MB. The assumption that they were not in that location prior to lysis requires their movement into the narrowest cell dimension, at the edge of the cell in the plane of flattening. Since the dimensions of a centriole pair (even without associated microtubules) approach that of the cell at this edge, such movement is highly unlikely. (b) In detergent-lysed erythrocytes of several other vertebrates which are known to contain centrioles, centrioles are not found distributed through the cytoplasmic region, but remain adjacent to the nucleus (Euteneuer *et al.,* 1985). (c) Immediately after cell lysis with Triton in both skate (this paper) and blood clam erythrocytes (Nemhauser *et al.,* 1983), without chemical fixation, centrioles are observed to be fixed in position. (d) When non-mammalian erythrocytes are lysed with Triton, the cell surface-associated cytoskeletal network (SAC; visible in Figs. 4, 5, 7) collapses inward from both sides as hemoglobin and other soluble components are released, trapping organelles within. This would be expected to prevent or at least inhibit marked movement of centriole pairs with associated microtubules. (e) In one experiment on the skate eryth-

rocytes, glutaraldehyde was included in the Triton lysis medium, so that fixation would proceed simultaneously with cell lysis. Centriole distribution in these cytoskeletons was similar to that observed in the unfixed cytoskeletons (skate #6 *vs.* skates #1–5, Table I).

Although the observations on MB-associated centrioles reported here are probably the first for circulating erythrocytes of a vertebrate, the hemoglobin-containing erythrocytes of "blood clams" and certain sea cucumbers constitute well-documented prior cases among the invertebrates (Fontaine and Lambert, 1973; Nemhauser *et al.,* 1983). In the case of the blood clams, there is one centriole pair closely associated with each MB, and microtubule nucleating activity during experimentally induced MB reassembly initiates at, or in the vicinity of, these centrioles (Cohen and Nemhauser, 1980; Nemhauser *et al.,* 1983). The skate also has a large number of MB-associated centrioles, but the situation differs from that in blood clams. First, centrioles are not associated with every MB; as observed in cytoskeletons, they frequently occur distant to it in the open cytoplasmic region or adjacent to the nucleus. Second, there is considerable variation in centriole distribution in the erythrocytes of different skates (Table I). These observations are consistent with the following possibility: all vertebrate MBs may initially have centrioles/centrosomes associated as microtubule organizing centers during normal erythrocyte differentiation. As cells mature, centrioles might redistribute such that their location is a function of erythrocyte age. This could account for variation in numbers of MB-associated centrioles in different animals, reflecting erythrocyte age distribution in the circulating population and variations in hematopoietic activity prior to the time of blood sampling. A similar scenario has been suggested recently by Euteneuer *et al.* (1985) on the basis of data on MB microtubule polarity.

Although the centrioles are not randomly distributed in skate erythrocytes, centriole function with respect to the MB obviously remains to be demonstrated in this species. Unfortunately, under conditions tested thus far, the MBs are not cold-labile in living cells of *R. erinacea.* Thus it has not yet been possible to examine the role of centrioles in MB reassembly as done previously in the case of blood clam erythrocytes (Nemhauser *et al.,* 1983). However, the morphology of the pointed cytoskeletons (Fig. 8) is suggestive of centriole activity. Although only two such skate cytoskeletons have been observed to date, their appearance is similar to that observed in native pointed cytoskeletons of blood clam erythrocytes, and to stages in blood clam MB reassembly (Nemhauser *et al.,* 1983), in which centrioles and associated material constitute the nucleating "pole" for the MB. Is pointed morphology a normal stage in erythrocyte differentiation in the non-mammalian vertebrates? The literature does not answer this question, but pointed cells have been observed in the circulation of adult skates (Fig. 1), chickens (Lucas and Jamroz, 1961), and several other vertebrates.

In examining the possible role of centrioles in MB biogenesis, attention must be paid to possible differences between experimentally induced MB reassembly in mature erythrocytes and normal MB biogenesis during erythrocyte differentiation. Although centrosomes appear to be active in initial stages of MB biogenesis in chick bone marrow erythroblasts, they do *not* participate in experimentally induced MB reassembly in mature chicken erythrocytes (Miller and Solomon, 1984; Swan and Solomon, 1984; Murphy *et al.,* 1986). In addition, the MB in differentiating chicken erythroblasts contains approximately 50 microtubules, whereas that in mature chicken erythrocytes contains only about 12, and similar differences in microtubule numbers in differentiating *versus* differentiated erythrocytes have been observed for other species (Small and Davies, 1972; Yamamoto and Iuchi, 1975; Miller and Solo-

mon, 1984). Thus, experimentally induced MB reassembly in a morphologically differentiated cell can provide only limited information about MB biogenesis. The role of centrioles and pericentriolar material in MB biogenesis must be properly assessed directly in the normally differentiating erythroblasts of various species, during the morphological transition from mitotic cell to flattened erythrocyte.

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